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COMMENTARY

Background Information

All hybridization methods depend upon the ability f denatured DNA to reanneal when complementary strands are present in an environment near but below their T_m (melting temperature). In a hybridization reaction involving double-stranded DNA on a filter and a single-stranded DNA probe there are three different annealing reactions occurring. First, there are the desired probe-DNA interactions which result in signal. Second, there are mismatch interactions that occur between related but nonhomologous sequences; these mismatch hybrids are the ones that must be eliminated during the washing of the filters. Non-sequence-specific interactions also occur and these result in noise. The ability to extract information from a particular hybridization experiment is a function of the signal-to-noise ratio. High background or poor specific signal can both result in uninterpretable results.

Washing nitrocellulose filters is required to remove excess radioactive probe, as well as radioactive probe that has bound to the DNA on the filter as mismatch hybrids. Temperature and salt concentration dramatically affect the maintenance of specific hybrids. Detergents and other charged species can have a profound effect upon the nonspecific binding of species that contribute to background. In this protocol, hybridization is achieved in a solution containing 50% formamide. Excess probe is rinsed away under low-stringency conditions so that further hybridization will not occur. Once the hybridization solution is rinsed away, it is possible to proceed to a high-stringency wash without fear of further hybridization. When washing is complete, the filters should produce very little "noise" when monitored with a Geiger counter. Although single-copy sequence probe normally does not produce a signal that is detectable with a Geiger counter, a probe corresponding to more abundant sequences will produce a signal that can be "heard" with a Geiger counter.

Literature Review

Hybridization to filter membranes forms a basis of recombinant DNA technology and is described in detail earlier in the manual (*UNIT 2.9*). The protocols described here vary from those used for Southern blot filter hybridization in that the volume of the hybridization is usually larger and the washing conditions are different. Dextran sulfate is an important component of the hybridization solution as it increases the rate of reassociation of the nucleic acids.

The protocols in this unit describe methods for hybridizing radioactive probes to membrane-bound plaques or colonies. These procedures for screening recombinant clones were first suggested by Grunstein and Hognes (1975) and by Benton and Davis (1977). The conditions of hybridization proposed in the basic protocol involving hybridization in formamide was originally described by Denhardt (1966) and Gillespie and Spiegelman (1965) while the alternate protocol using aqueous hybridization solution was introduced by Church and Gilbert (1984).

The method of washing filters under stringent conditions to remove background was first proposed by Southern (1975). Botchan et al. (1976) described the benefit of adding SDS to the wash solution. Jeffreys and Flavell (1977) first employed the wash conditions described in the protocols presented here.

Critical Parameters

Hybridization. Kinetically, the hybridization of DNA (or RNA) probes to filter-bound DNA is not significantly different from hybridization in solution. For single-stranded probes, the rate of hybridization follows first-order kinetics, since probe is available in excess. Under conditions of excess probe, the time for hybridization is inversely proportional to the probe concentration. For double-stranded probes the rate of hybridization displays a more complex relationship to the initial probe concentration. However, to a first approximation the initial probe concentration is inversely proportional to the rate of hybridization. To determine the actual time required for the successful hybridization of a given probe, either empirical data must be available or the following formula can be used to determine the length of time (in hours) required to achieve 50% hybridization (T_{50}):

$$1/x \times y/5 \times z/10 \times 2 = T_{50}$$

where x is the weight of probe in micrograms; y is the complexity of probe in kilobases; and z is the volume of hybridization solution in milliliters. The length of time T is given in hours. Maximum hybridization signal will be obtained if the reaction is allowed to proceed to $5 \times T_{50}$, although 1 to $2 \times T_{50}$ is often used.

It is also clear that nonspecific interactions

occur and that in any hybridization, sources of noise will be present. Therefore, from a practical standpoint one conventionally utilizes concentrations of nick-translated probe n the order of 1 to 15 ng/ml of hybridization, where the specific activity of the probe is from 5×10^7 cpm/μg to $>10^8$ cpm/μg. Too much probe in a hybridization is as bad as too little.

One important source of background hybridization to filters is due to the hybridization of the probe to vector sequences or to *E. coli* DNA. Be certain that there is no vector or *E.coli* DNA sequences in the probe. This can best be ensured by isolating the probe from one type of vector (e.g., plasmid) and screening a library made with a different type of vector (e.g., bacteriophage).

Washing temperature. Washing at low stringency is a straightforward proposition. Buffer is added at room temperature and washing proceeds at room temperature.

High-stringency wash is determined empirically. The relative homology between the probe and target sequence is a determining parameter. If the homology is 100%, a high temperature (65° to 75°C) can be used. As the homology drops, lower washing temperatures must be used. In general one starts at 37° to 40°C, raising the temperature by 3° to 5°C intervals until background is low enough not to be a major factor in the autoradiography.

The length of the probe is also important. Very short probes (<100 bp) must be washed at lower temperatures, even if the homology is 100%. Washing strategy is the same as for probes of differing homology.

Salt concentration. The lower the salt concentration, the higher the stringency. With this said, the protocols as outlined do not require adjustment of salt concentration for adjustment of stringency. Only the washing temperature is varied.

Probe. The nucleic acid probe must be of high specific activity and greater than 50 bp in length so that it can form stable hybrids.

Anticipated Results

After washing the filters the background should be barely detectable with a Geiger counter.

With a high-specific-activity probe $>5 \times 10^7$ cpm/μg and an overnight hybridization reaction with a 1-kb unique sequence probe, hybridizing bacterial colonies or bacteriophage plaques can be visualized after a 1 to 18 hr exposure.

Time Considerations

Generally hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots. However, with probes of increasing complexity longer hybridization times are required. This is preferable to increasing the probe concentration from experiment to experiment.

Autoradiography requires 1 to 18 hr.

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9. Develop the films; if a high background prevents proper interpretation of the films, rewash the filters at a higher temperature.

10. Number and mark the orientation of the films as described in *UNIT 6.3*. Spots that appear in precisely the same place on duplicate filters are "positives" (winners) and should be processed as described in *UNIT 6.5*.

    It is impossible to identify the characteristics of a true positive spot. Only colonies or plaques that produce evidence of hybridization on both filter copies should be processed as described below. Note that the intensity of the spot can vary dramatically between the duplicate filters. If a clear-cut spot appears on one filter and only a darkening of the background appears on the other, this should be considered positive and the plate should be processed as described in UNIT 6.5.

    Note that if two different oligonucleotide mixtures representing two different parts of the protein are available, either the positives obtained with one probe can then be hybridized with the other probe or four filter copies of the library can be made and hybridized to the two probes. Of course, depending on how far apart the sequences that hybridize to the two probes are, it is possible that neither will be present on a less than full-length cDNA clone.

BASIC PROTOCOL

HYBRIDIZATION IN TETRAMETHYLAMMONIUM CHLORIDE (TMAC)

This procedure is similar to the SSC protocol except that hybridization and washing are performed in solutions containing TMAC. In TMAC, the melting temperature of an oligonucleotide is a function of length and is independent of base composition; thus, spurious hybridization due to high G-C content of some of the oligonucleotides is reduced. Conditions are described for using 17-base oligonucleotides, but information is provided for determining the conditions when oligonucleotides of various lengths are employed.

Materials

Nitrocellulose or nylon membrane filters bearing plasmid, bacteriophage, or cosmid libraries (*UNITS 6.1* and *6.2*)
150-mm LB agarose plates (*UNIT 1.1*), prewarmed to 37°C
2× SSC/0.5% SDS/50 mM EDTA, pH 8.0, prewarmed to 50°C
TMAC hybridization solution, prewarmed to hybridization temperature
TMAC wash solution
2× SSC/0.1% SDS

15-cm glass crystallizing dishes
Filter forceps (e.g., American Scientific Products #2568-1)

Additional reagents and equipment for autoradiography (*APPENDIX 3*)

Process and prehybridize the filters

1. Process filters bearing bacterial colonies as described in *UNIT 6.2*. Produce filters bearing amplified bacteriophage plaques as follows:

   a. Plate the bacteriophage from the library on LB agarose plates and transfer to nitrocellulose filters as described in *UNIT 6.1*, steps 1 to 7.

      To obtain maximum sensitivity with oligonucleotide probes when the amplification procedure is used, plating density should be reduced to 8,000 to 10,000 plaques per 150-mm plate.

      Either nitrocellulose or nylon (Colony/Plaque Screen Filters by New England Nuclear) filters can be used in this procedure. Nitrocellulose filters become fragile when hybridized in TMAC and must be handled very carefully. If this becomes a problem and nylon filters are substituted, the phage plaques must be amplified overnight. The rest of the protocol is unchanged.

b. Amplify the bacteriophage by transferring the wet filter to a prewarmed (37°C) LB agarose plate so that the surface bearing the bacteriophage is faceup.

Refrigerate the master plates upon which the recombinant phage library were plated to prevent any further plaque expansion.

c. Incubate the plates at 37°C until the bacterial lawn re-forms on the surface of the nitrocellulose and plaques are evident. Plaque size will be somewhat larger than those on the original plate. This usually requires a 5- to 12-hr incubation period. Longer periods of growth will produce a dense bacterial lawn without significantly increasing plaque size or affecting hybridization signal.

Bacteriophage that produce small plaques (e.g., EMBL) are usually plated in the evening and allowed to grow overnight. The plaques are transferred to nitrocellulose filters the following morning and the phage are amplified on the filters by incubation for 5 to 7 hr during the day. Phage that produce large plaques (e.g., λgt10) are plated early in the morning, allowed to grow 5 to 7 hr, transferred to nitrocellulose filters (steps 6 and 7 of UNIT 6.1), transferred to fresh plates, and then incubated for amplification overnight.

d. Denature and bind the bacteriophage DNA to nitrocellulose filters as described in steps 8 to 11 of *UNIT 6.1*.

2. Wash filters bearing bacterial colonies as described in step 1 of the SSC protocol. Wet bacteriophage-bearing filters in a prewarmed (50°C) solution of 2× SSC/0.5% SDS/50 mM EDTA (pH 8.0). Float the filters on top of the solution (with the surface containing the dried bacteria and plaques faceup) to allow the nitrocellulose to wet completely. Submerge the filters and, with a gloved hand, gently rub the surface of the filters to remove the dried bacterial debris. Transfer the filters to a container of fresh solution of 2× SSC/0.5% SDS/50 mM EDTA to remove bacterial debris.

Alternatively, the filters can be incubated in this solution at 65°C for one to several hours and then scrubbed. Inadequate scrubbing of the filters results in an increase of nonspecific background hybridization, obscuring positive hybridization signals in the subsequent screening procedure.

3. Transfer the filters to a 15-cm glass crystallizing dish containing 5 to 10 ml TMAC hybridization solution (per filter), which has been prewarmed to the appropriate hybridization temperature (48°C for 17-mer oligonucleotides; see Fig. 6.4.1 and commentary for other oligonucleotides) and seal the dishes with plastic wrap and rubberbands. Prehybridize 1 to 2 hr at the hybridization temperature, which is 5° to 10°C below the melting temperature.

Prehybridization and hybridization can be performed in glass crystallizing dishes that are slightly larger in diameter than the nitrocellulose filters. Gentle agitation on an orbital platform shaker will allow the solution to pass freely between the stacked filters and prevent the filters from sticking together. Place no more than 25 to 30 filters in each dish.

Alternatively, prehybridization and hybridization can be performed in a sealable bag (see SSC protocol) with <10 filters per bag.

Hybridize oligonucleotides to the filters

NOTE: The following hybridization and wash temperatures have been optimized for the use of oligonucleotide probes of 17 nucleotides in length. If different length oligonucleotide probes are used, these conditions should be adjusted based on the information presented in the commentary.

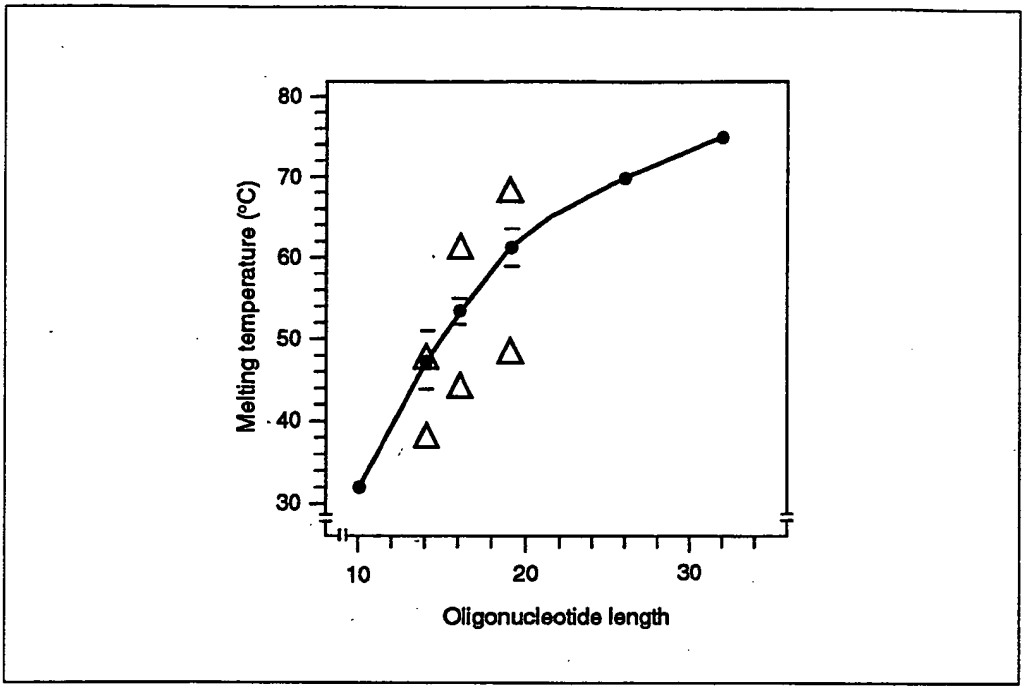

Figure 6.4.1 Melting temperatures of oligonucleotides of different length in TMAC and SSC hybridization solutions. Dots represent the average melting temperature of several different oligonucleotides of length 14, 16, or 19 bases in TMAC; bars represent the high and low melting temperatures for each length. Triangles represent the high and low melting temperatures for the same oligonucleotides in SSC. The melting temperature of only one oligonucleotide of length 10, 26, or 32 bases was determined. Hybridization temperature should be 5° to 10°C below the melting temperature, and washing temperature also should be 5° to 10°C below the melting temperature (Jacobs et al., 1988).

4. Transfer filters to a hybridization vessel (or bag) containing fresh, prewarmed (48°C) TMAC hybridization solution to remove residual SSC and bacterial debris from step 2 and to restore TMAC concentration to 3 M. Use 5 to 10 ml TMAC hybridization solution per filter.

5. Add 1 to 2×10^6 cpm of ^{32}P-labeled oligonucleotide probe per ml of hybridization solution directly to the solution of step 4 and incubate 40 to 60 hr at 48°C with gentle agitation on an orbital shaker.

   ^{32}P-label the oligonucleotide(s) with T4 polynucleotide kinase as described in the support protocol.

Wash the filters

6. Discard the hybridization solution containing radioactively labeled probe and rinse the filters with TMAC wash solution at room temperature. Use 5 to 10 ml TMAC wash solution per filter.

7. Transfer the filters individually to fresh TMAC wash solution (200 to 250 ml) and wash 15 min at room temperature with gentle agitation.

   Individual transfer of filters reduces background.

8. Replace the room-temperature TMAC wash solution with a similar volume of prewarmed TMAC wash solution and incubate the filters 1 hr at the appropriate wash temperature (50°C for 17-mers; see Fig. 6.4.1 and commentary).

9. Replace the TMAC wash solution with a similar volume of 2× SSC/0.1% SDS. Wash the filters 10 min at room temperature.

10. Repeat step 9 twice.

These washes remove residual traces of TMAC from the nitrocellulose filters and prevent crystallization of the TMAC salts upon drying.

Perform autoradiography

11. Autoradiograph the filters as described in steps 8 to 10 of the SSC protocol.

LABELING THE 5′ ENDS OF MIXED OLIGONUCLEOTIDES

SUPPORT PROTOCOL

A mixture of synthetic oligonucleotides is ^{32}P-labeled using T4 polynucleotide kinase. This protocol is similar to that used to label a single oligonucleotide (*UNIT 3.10*).

Materials

2.5 to 250 pmol mixed oligonucleotides
[γ-^{32}P]ATP (>7000 Ci/mmol)
25 to 50 U T4 polynucleotide kinase (*UNIT 3.10*) and 10× kinase buffer (*UNIT 3.4*)
Ice-cold 10% trichloroacetic acid (TCA)

1. Set up reaction mixture on ice in microcentrifuge tube as follows:

2.5 to 250 pmol mixed oligonucleotides
7.5 µl 10× T4 polynucleotide kinase buffer
66 pmol [γ-^{32}P]ATP (200 µCi)
25 to 50 U T4 polynucleotide kinase
H_2O to 75 µl

Incubate 30 min at 37°C.

The reaction mixture should have either equimolar amounts of label and oligonucleotide ends, or the label should be in molar excess.

1 mol deoxyribonucleotide ≅ 330 g
1 OD_{260} ≅ 40 µg/ml oligonucleotide
1 µg 14-base oligonucleotide ≅ 0.24 nmol
1 µg 17-base oligonucleotide ≅ 0.18 nmol
1 µg 20-base oligonucleotide ≅ 0.15 nmol

150 pmol

2. At the end of the reaction, check for incorporation of label by precipitating 1 µl of a diluted aliquot with ice-cold 10% TCA (acid precipitation, *UNIT 3.4*) and counting the incorporated radioactivity.

Using equimolar amounts of oligonucleotide and label, ~30% to 90% of the counts are incorporated.

The labeled oligonucleotide can be further purified by a combination of phenol extraction and/or ethanol precipitation (UNIT 2.1). To remove unincorporated label, oligonucleotides of 17 bases or longer can be quantitatively precipitated from a solution of 2.5 M ammonium acetate containing 25 µg carrier DNA plus 9 vol of 100% ethanol. The resulting pellets are washed with 70% ethanol, followed by 95% ethanol, air dried, and resuspended in 100 µl TE buffer.

3. Store mixture in appropriate container at –20°C.

REAGENTS AND SOLUTIONS

Prehybridization solution

6× SSC (*APPENDIX 2*)
5× Denhardts solution (*APPENDIX 2*)
0.05% sodium pyrophosphate
100 µg/ml boiled herring sperm DNA
0.5% sodium dodecyl sulfate (SDS)

SSC hybridization solution

6× SSC (*APPENDIX 2*)
1× Denhardt's solution (*APPENDIX 2*)
100 μg/ml yeast tRNA
0.05% sodium pyrophosphate

TMAC hybridization solution

3 M tetramethylammonium chloride (see recipe below for stock solution)
0.1 M $NaPO_4$, pH 6.8
1 mM EDTA, pH 8.0
5× Denhardt's solution (*APPENDIX 2*)
0.6% SDS
100 μg/ml denatured salmon sperm DNA

TMAC wash solution

3 M tetramethylammonium chloride (see recipe below for stock solution)
50 mM Tris·Cl, pH 8.0
0.2% SDS

Tetramethylammonium chloride (TMAC), 6 M stock solution

Dissolve 657.6 g TMAC (mol wt = 109.6) in H_2O and bring to 1 liter. Filter the solution through Whatman No. 1 filter paper and determine the precise concentration of the solution by measuring the refractive index (n) of a 3-fold diluted solution. The molarity (M) of the diluted solution = $55.6(n - 1.331)$ and the molarity of the stock solution = 3 × M. TMAC can be stored at room temperature in brown bottles.

CAUTION: *TMAC can irritate eyes, skin, and mucous membranes. It should be used with adequate ventilation in a fume hood. Used TMAC solutions should be collected and discarded as hazardous and/or radioactive waste. Small amounts (<10 ml) can be flushed down the drain with a large quantity of tap water.*

COMMENTARY

Background Information and Literature Review

Based on a study of the effect of single-bp mismatches on the hybridization behavior of oligonucleotides to ΦX174 DNA, Wallace et al. (1979) proposed that synthetic oligonucleotide mixtures representing all the possible coding sequences for a particular peptide sequence might be used as specific probes to identify cloned DNA. They demonstrated that duplexes with a single-bp mismatch—formed when 11-, 14-, or 17-base oligonucleotides were hybridized to ΦX174 DNA—were significantly less stable (dissociated at lower temperatures) than their perfectly matched counterparts. This difference in thermal stability made it possible, by the appropriate choice of hybridization conditions, to virtually eliminate the formation of mismatched duplexes without affecting the formation of perfectly matched ones. Mixed-sequence oligonucleotide probes were first used successfully for the isolation of a cloned cDNA encoding human β_2-microglobulin (Suggs et al., 1981). Many different cDNA clones have been isolated using this approach.

Recombinant DNA libraries can be screened with probes consisting of single oligonucleotides or mixtures of oligonucleotides. Bacteriophage or plasmid clones that encode sequences perfectly complementary to the probe must be distinguished from clones that do not. Most often recombinant DNA libraries are screened with probes consisting of multiple oligonucleotides, chosen to cover all the coding possibilities of a particular amino acid sequence. Since any member of the oligonucleotide pool could match the target sequence, hybridization and wash temperatures are selected that allow the oligonucleotide with the lowest melting temperature (the lowest G-C content) to hybridize efficiently. In solutions containing SSC, oligonucleotides of high G-C content can potentially form stable duplexes with sequences to which they are not perfectly complementary.

Mixed probes can produce a high number of false positives using the SSC hybridization

conditions. This problem can be minimized by replacing SSC with TMAC in the hybridization and wash solutions. The melting temperature of long, native DNA in 3 M TMAC is indep n-dent of base composition (Melchior and von Hippel, 1973) and rate of renaturation is approximately the rate of renaturation in SSC (Wetmur, 1976). In solutions containing 3 M TMAC, the thermal stability of oligonucleotides 16 bases or longer is sequence-independent (Wood et al., 1985; Jacobs et al., 1988), and non–Watson-Crick base pairs decrease the thermal stability of oligonucleotide duplexes 1° to 1.5°C per percent mismatch (Jacobs et al., 1988). Thus, when the hybridization and wash are performed in 3 M TMAC the appropriate temperature is defined by the length of the oligonucleotide probes. All of the oligonucleotides in the pool will hybridize with equal efficiency to their complementary sequences and with reduced efficiency to sequences to which they are not perfectly complementary. The gene encoding erythropoietin was isolated using TMAC (Jacobs et al., 1985).

The support protocol describes a procedure for ^{32}P-labeling synthetic oligonucleotides and is adapted from Richardson (1971).

Critical Parameters

In choosing the stretch of amino acids to be used as the blueprint for the mixture of oligonucleotides, one finds a stretch of amino acids that is encoded by a minimum number of possible codons. Unfortunately, most amino acids are encoded by two or four codons. Only methionine and tryptophan—among the rarest of amino acids—are encoded by a single codon. In addition, three of the commonest amino acids have a total of six different codons each (see codon chart, *APPENDIX 1*). Computer programs are available to determine the optimal structure of oligonucleotide probes from amino acid sequences (Yang et al., 1984).

As the number of different sequences present in an oligonucleotide mixture increases, several possible problems arise. First, unfavorable signal-to-noise ratios may be a problem. However, mixtures of 17-bp oligonucleotides containing 300 to 600 different sequences have been used. Second, as the number of different sequences increases, the probability of completely matching an unrelated and undesired mRNA sequence increases. Third, the distinct possibility remains that the sequence of interest may not be present, and/or incorrect oligonucleotides may be present in the mixture.

Another strategy is to use a single, long, synthetic oligonucleotide probe selected from codon usage frequencies (reviewed by Lathe, 1985). The uncertainty at each codon is ignored and increased probe length is used to confer probe specificity. The choice of the codon for each amino acid is based on codon utilization data, intercodon dinucleotide frequencies, and other rules. Knowledge of dinucleotide frequencies is important because of the decreased frequency of the dinucleotide pair, CpG. A list of codons recommended by Lathe (1985) is given in Table 6.4.1.

Determining the temperatures for hybridizing and washing is important to reduce the number of false positive plaques or colonies. Although there are examples of isolating clones that have a one-base mismatch with the oligonucleotide probe (<23 nucleotides), this usually is the result of a wrong sequence being

Table 6.4.1 Optimum Codon Choice When Deducing a Probe Sequence from Human Amino Acid Sequence Data

	Optimum codon[a] when subsequent codon begins with	
Amino acid	A or C or T	G
Methionine	ATG	nc[b]
Tryptophan	TGG	nc
Tyrosine	TAC	TAT
Cysteine	TGC	TGT
Glutamine	CAG	nc
Phenylalanine	TTC	TTT
Aspartic acid	GAC	GAT
Asparagine	AAC	AAT
Histidine	CAC[c]	CAT
Glutamic acid	GAG	nc
Lysine	AAG	nc
Alanine	GCC	GCT
Isoleucine	ATC	ATT
Threonine	ACC	ACA[f]
Valine	GTG[d]	nc
Proline	CCC[e]	CCT
Glycine	GGC	nc
Leucine	CTG	nc
Arginine	CGG	nc
Serine	TCC	TCT

[a]The optimum codon is the most frequent codon in all cases except Arg and Ser, where the indicated triplets generate a higher overall homology to all possible codons. Reprinted with permission from *Journal of Molecular Biology.*

[b]No change.

[c]CAT when followed by C.

[d]GTC when followed by T.

[e]CCA when followed by T.

[f]These cases do not follow the "replace C by T" rule applied when the subsequent codon is headed by G.

present in the oligonucleotide mixture. Furthermore, one occasionally synthesizes a mixture of oligonucleotides that is missing the single correct sequence. This possibility should be remembered if no positive results are obtained after screening a library.

Using two copies of each filter to compare the signal obtained with both is an important method for eliminating false positive clones. Because of the variation in the appearance of positive signals and the frequently high backgrounds, it is virtually impossible to tell if a signal from one filter represents true oligonucleotide hybridization. Libraries can be screened in duplicate with one oligonucleotide pool or can be screened with two different oligonucleotides derived from amino acid sequences for the same protein.

The SSC hybridization procedure is similar to that presented by Woods et al. (1982). Other procedures use Tris buffers instead of sodium citrate buffers in the hybridization solution and employ a shorter period of time for the final stringent wash (Connor et al., 1983).

In the SSC protocol, a low temperature for hybridizing the oligonucleotide to the filters is critical to allow maximal binding of the perfectly complementary oligonucleotides. A high temperature in the stringent wash is essential to remove mismatch hybrids. The method for optimizing the signal-to-noise ratio is to wash the hybridized filters at an empirically determined temperature. Initial hybridization and stringent washing conditions can be determined by formulas (see Lathe, 1985) and improved upon by trial-and-error adjustments. Generally, probes ≥50 bp long that have ≥80% homology will be specific and will hybridize to the sequence of interest. The probability of constructing an appropriate oligonucleotide probe is high but there is a possibility that a nonhybridizing probe will be made (Parker et al., 1986).

In the SSC protocol, background level of radioactivity can vary between none (unusual) to dark gray (usually not a problem). If the background is high, positive spots can often be identified by comparing duplicate filters. Filters can be rewashed at a higher temperature and reexposed to film if the background is too dark. If this does not remove the high background, too little hybridization solution was used or the oligonucleotide probe is no good and should be remade. Another possibility is that the probe is hybridizing to *E. coli* DNA.

The TMAC protocol describes hybridization times and temperatures appropriate for screening libraries with 17-mers. In practice we find that using both hybridization and wash temperatures in the range of 5° to 10°C below the melting temperature (T_m) for an oligonucleotide of particular length gives optimal results. With increasing degeneracy of the oligonucleotide, it is advisable to use the lower range of hybridization and wash temperatures (10°C below T_m) because of the reduced signal. For other applications or when using oligonucleotides of different lengths and complexity, the appropriate conditions can be determined using the information in Figure 6.4.1. A plot of T_m versus oligonucleotide length in TMAC and SSC is shown in Figure 6.4.1. Clearly, there is a broader melting range in SSC than in TMAC. By referring to this figure, the T_m for an oligonucleotide from 16 to 32 bases can be determined. Alternatively, the following equation can be used:

$$T_m = -682(L^{-1}) + 97$$

where L is the number of bases in the oligonucleotide and T_m is °C.

With TMAC, background level due to radioactivity occurs sometimes with particular oligonucleotides but has not obscured duplicate positives. If oligos shorter than 17 bases are used, the general background will be higher, presumably because of the lower temperature used. If the background is too high, the filters can be washed at 5°C below the T_m, but only in preequilibrated buffer and for no more than 10 to 15 min (steps 7 to 10 of TMAC protocol).

When bacteriophage plaques were amplified and probes hybridized in TMAC, 17-mers that were 600-fold degenerate were used to isolate clones from a λgt10 cDNA library; hybridization was performed for 3 days and exposure times of 24 hr were sufficient. Genomic libraries have been screened with 17-mers that were 512-fold degenerate, requiring hybridization times of 2 to 3 days and exposure times of 1 to 2 days. These times were not rigorously determined. For longer oligonucleotides or with less complex pools, shorter hybridization times may be used. Exposure times are usually determined by developing a few films and making a judgement based on the background and number of positives. When the bacteriophage plaques are amplified, stripping and screening the filters a second time is not recommended.

Anticipated Results

Any recombinant DNA library screened with oligonucleotides will yield "positives" because statistically there will be sequences that are